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**Authors**

Liu, Hsien-Lin

Wang, Christine H-T

Chiang, En-Pei Isabel

et al.

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**RESEARCH**

# Tryptophan plays an important role in yeast's tolerance to isobutanol

Hsien-Lin Liu[1,2,3], Christine H.-T. Wang[2], En-Pei Isabel Chiang[4,6], Chieh-Chen Huang[1,3,6*] and Wen-Hsiung Li[1,2,5*]

## Abstract

**Background:** Isobutanol is considered a potential biofuel, thanks to its high-energy content and octane value, limited water solubility, and compatibility with gasoline. As its biosynthesis pathway is known, a microorganism, such as *Saccharomyces cerevisiae*, that inherently produces isobutanol, can serve as a good engineering host. Isobutanol's toxicity, however, is a major obstacle for bioproduction. This study is to understand how yeast tolerates isobutanol.

**Results:** A *S. cerevisiae* gene-deletion library with 5006 mutants was used to screen genes related to isobutanol tolerance. Image recognition was efficiently used for high-throughput screening via colony size on solid media. In enrichment analysis of the 161 isobutanol-sensitive clones identified, more genes than expected were mapped to tryptophan biosynthesis, ubiquitination, and the pentose phosphate pathway (PPP). Interestingly, adding exogenous tryptophan enabled both tryptophan biosynthesis and PPP mutant strains to overcome the stress. In transcriptomic analysis, cluster analysis of differentially expressed genes revealed the relationship between tryptophan and isobutanol stress through some specific cellular functions, such as biosynthesis and transportation of amino acids, PPP, tryptophan metabolism, nicotinate/nicotinamide metabolism (e.g., nicotinamide adenine dinucleotide biosynthesis), and fatty acid metabolism.

**Conclusions:** The importance of tryptophan in yeast's tolerance to isobutanol was confirmed by the recovery of isobutanol tolerance in defective strains by adding exogenous tryptophan to the growth medium. Transcriptomic analysis showed that amino acid biosynthesis- and transportation-related genes in a tryptophan biosynthesis-defective host were up-regulated under conditions similar to nitrogen starvation. This may explain why ubiquitination was required for the protein turnover. PPP metabolites may serve as precursors and cofactors in tryptophan biosynthesis to enhance isobutanol tolerance. Furthermore, the tolerance mechanism may also be linked to tryptophan downstream metabolism, including the kynurenine pathway and nicotinamide adenine dinucleotide biosynthesis. Both pathways are responsible for cellular redox balance and anti-oxidative ability. Our study highlights the central role of tryptophan in yeast's isobutanol tolerance and offers new clues for engineering a yeast host with strong isobutanol tolerance.

**Keywords:** Isobutanol, Tryptophan, Gene-deletion library screening, Transcriptomic analysis

## Background

Using renewable feedstock to produce energy can reduce the usage of petroleum. Indeed, bio-alcohols are potential gasoline replacements. Among them, isobutanol, a branched-chain alcohol, is superior to ethanol in properties such as a higher energy content, limited solubility in water and no stress cracking in pipelines. In addition, isobutanol has a higher octane number than the

*Correspondence: cchuang@dragon.nchu.edu.tw; whli@uchicago.edu
[1] Program in Microbial Genomics, National Chung Hsing University and Academia Sinica, Taipei, Taiwan
Full list of author information is available at the end of the article

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linear *n*-butanol and it allows blending with gasoline in a greater amount due to its lower oxygen content than that of ethanol. Moreover, its gasoline compatibility allows the use of existing infrastructure and combustion engines [1, 2].

For bio-based production of isobutanol, *Saccharomyces cerevisiae* has the advantages of tolerance to harsh fermentation environment, immunity to phage contamination, and simple separation. Furthermore, as *S. cerevisiae* has been commonly used in the current bio-ethanol production, the same host organism facilitates a simple transition to isobutanol production with existing fermentation facilities. *S. cerevisiae* inherently produces isobutanol through the valine Ehrlich degradation pathway. Moreover, the well-established genetic tools in *S. cerevisiae* and modern synthetic biology techniques allow for the engineering of yeast hosts for bioproduction of chemicals. Many efforts have been made toward yeast engineering for isobutanol production [3–5]. However, as the production of a novel compound creates a stress to the host, it becomes a hurdle in mass production [6]. To solve this problem, genetic modifications have been made to obtain tolerant strains [7, 8]. Our previous study has provided an example for increasing ethanol and *n*-butanol productivity through enhanced tolerance of the host cell by removing oxidative stress in *E. coli* [9]. As isobutanol toxicity is a major constraint on bioproduction, understanding and improving microbial tolerance to isobutanol has become a hot topic [10]. Kuroda et al. [10] screened a gene-deletion library and discovered the importance of the pentose phosphate pathway (PPP) and tryptophan biosynthesis for yeast isobutanol tolerance. Moreover, deletion of GLN3, a transcriptional activator nitrogen catabolite repression system, was found to increase isobutanol tolerance by blocking the nitrogen starvation signal induced by isobutanol, and the yield of isobutanol was therefore increased [10].

To further understand the mechanism of yeast isobutanol tolerance, we employed a gene-deletion library with 5006 mutants of *S. cerevisiae* (YKO MATa Strain Collection-Glycerol Stocks, Catalog #: YSC1053) and solid-based colony image screening to identify yeast genes and metabolic pathways related to isobutanol tolerance. Enrichment analysis was then employed to categorize the isobutanol-sensitive genes in terms of their functions, and tryptophan biosynthesis, ubiquitination, and the pentose phosphate pathway (PPP) were found to be involved in isobutanol tolerance. To test the importance of tryptophan biosynthesis, we studied whether exogenous tryptophan can recover isobutanol tolerance of tryptophan biosynthesis-mutant strains. Finally, transcriptomic analysis was conducted to investigate possible molecular mechanisms underlining yeast's isobutanol

tolerance. Cluster analysis of differentially expressed genes (DEGs) was applied to infer the correlation between cellular responses and to map the relationship between tryptophan and isobutanol stress onto specific functions. Our study offers an integrated view for the role of tryptophan in yeast's isobutanol tolerance.

## Results

### Screening for genes and pathways related to isobutanol tolerance

In order to identify genes related to isobutanol tolerance, an *S. cerevisiae* gene-deletion library (YKO MATa Strain Collection-Glycerol Stocks, Catalog #: YSC1053) was used for high-throughput screening. Each mutant strain was inoculated on solid medium plates with and without isobutanol treatment. The colony size was quantified after the plate image was scanned and recognized by the software "ScreenMill" [11]. Statistical analysis of colony images identified a series of mutants sensitive to isobutanol. Among the 5006 mutant strains assayed, a total of 161 gene-deletion clones showed a significant growth-reduction (Additional file 1: Table S1). An enrichment analysis (DAVID Bioinformatics Resources 6.8) led to cell functions enriched among the 161 genes, where the top 3 categories were genes engaged in tryptophan biosynthesis, ubiquitination, and the pentose phosphate pathway (Fig. 1a). The involvement of ubiquitination in *n*-butanol tolerance has been reported by González-Ramos et al. [12]. Their evolved *n*-butanol-tolerant strains also showed isobutanol tolerance, implying that *n*-butanol and isobutanol may induce similar responses from yeast cells. The ubiquitination gene cluster of our enrichment analysis included DNA damage-responsive RNA polymerase-degradation factor *DEF1*, E2 ubiquitin-conjugating protein *UBC8*, SCF ubiquitin ligase complex subunit *GRR1*, SUMO-targeted ubiquitin ligase complex subunit *SLX5*, SUMO-targeted ubiquitin ligase complex subunit *SLX8*, anaphase promoting complex subunit *DOC1*, ubiquitin-specific protease *UBP13*, and ubiquitin-specific protease *UBP15*. The genes *UCC1* and *UMP1* involved in the ubiquitin–proteasome system were also identified by González-Ramos et al. [12]. Our data suggest that isobutanol and *n*-butanol share similar tolerance mechanisms involved in protein degradation.

Our enrichment analysis also revealed a key cluster of defective genes in the tryptophan biosynthesis pathway. Pentafunctional AROM protein (*ARO1*), chorismate synthase (*ARO2*), anthranilate synthase (*TRP2*), anthranilate phosphoribosyltransferase (*TRP4*), phosphoribosylanthranilate isomerase (*TRP1*), indole-3-glycerol-phosphate synthase (*TRP3*), and tryptophan synthase (*TRP5*) were identified. In addition, genes clustered in the pentose phosphate pathway (PPP) also contributed to isobutanol

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**(A)**

| Annotation Cluster 1 | Enrichment Score: 3.26 | G | | Count | P_Value | Benjamini |
|---|---|---|---|---|---|---|
| GOTERM_BP_DIRECT | aromatic amino acid family biosynthetic process | RT | | 7 | 1.0E-7 | 4.2E-5 |
| UP_KEYWORDS | Aromatic amino acid biosynthesis | RT | | 7 | 1.1E-7 | 1.8E-5 |
| GOTERM_BP_DIRECT | tryptophan biosynthetic process | RT | | 5 | 1.2E-6 | 2.6E-4 |
| UP_KEYWORDS | Tryptophan biosynthesis | RT | | 5 | 1.4E-6 | 1.0E-4 |
| KEGG_PATHWAY | Phenylalanine, tyrosine and tryptophan biosynthesis | RT | | 7 | 1.5E-6 | 6.3E-5 |
| KEGG_PATHWAY | Biosynthesis of amino acids | RT | | 11 | 5.3E-4 | 1.1E-2 |
| KEGG_PATHWAY | Biosynthesis of secondary metabolites | RT | | 17 | 1.1E-3 | 1.5E-2 |
| GOTERM_BP_DIRECT | tryptophan metabolic process | RT | | 3 | 1.5E-3 | 1.5E-1 |
| GOTERM_BP_DIRECT | cellular amino acid biosynthetic process | RT | | 8 | 6.2E-3 | 2.1E-1 |
| UP_KEYWORDS | Amino-acid biosynthesis | RT | | 8 | 7.0E-3 | 3.0E-1 |
| KEGG_PATHWAY | Metabolic pathways | RT | | 26 | 1.0E-2 | 8.1E-2 |
| KEGG_PATHWAY | Biosynthesis of antibiotics | RT | | 11 | 3.3E-2 | 2.1E-1 |
| GOTERM_BP_DIRECT | metabolic process | RT | | 12 | 5.6E-2 | 6.3E-1 |
| UP_KEYWORDS | Lyase | RT | | 6 | 7.4E-2 | 5.5E-1 |
| GOTERM_MF_DIRECT | lyase activity | RT | | 6 | 7.5E-2 | 8.9E-1 |
| GOTERM_MF_DIRECT | catalytic activity | RT | | 9 | 5.9E-1 | 1.0E0 |
| **Annotation Cluster 2** | **Enrichment Score: 1.81** | G | | Count | P_Value | Benjamini |
| GOTERM_BP_DIRECT | protein ubiquitination | RT | | 8 | 4.4E-3 | 1.7E-1 |
| UP_KEYWORDS | Ubl conjugation pathway | RT | | 9 | 1.6E-2 | 2.7E-1 |
| GOTERM_MF_DIRECT | ubiquitin-protein transferase activity | RT | | 6 | 5.4E-2 | 8.4E-1 |
| **Annotation Cluster 3** | **Enrichment Score: 1.78** | G | | Count | P_Value | Benjamini |
| KEGG_PATHWAY | Pentose phosphate pathway | RT | | 5 | 4.0E-3 | 4.2E-2 |
| GOTERM_BP_DIRECT | pentose-phosphate shunt | RT | | 4 | 4.2E-3 | 1.8E-1 |
| KEGG_PATHWAY | Biosynthesis of antibiotics | RT | | 11 | 3.3E-2 | 2.1E-1 |
| KEGG_PATHWAY | Carbon metabolism | RT | | 6 | 1.4E-1 | 5.4E-1 |

**(B)**

Abbreviation of metabolites
- (Ant) Anthranilate
- (Chr) Chorismate
- (DAHP) 3-deoxy-D-arabino-heptulosonate-7-phos-phate
- (DHQ) 3-dehydroquinate
- (DHS) 3-dehydroshikimate
- (E4P) Erythrose-4-phosphate
- (EPSP) 5-*enol*pyruvylshikimate-3-phosphate
- (F6P) Fructose 6-phosphate
- (G3P) Glyceraldehyde 3-phosphate
- (IGP) Indole-3-glycerol phosphate
- (PEP) Phosphoenolpyruvate
- (PRA) N'-(5'-phosphoribosyl) anthranilate
- (Ru5P) D-Ribulose 5-phosphate
- (R5P) D-Ribose 5-phosphate
- (Shk) Shikimate
- (S3P) Shikimate-3-phosphate
- (S7P) Sedoheptulose 7-phosphate
- (X5P) D-Xylulose 5-phosphate
- ($\alpha$-G6P) $\alpha$-D-Glucose-6-phosphate
- ($\beta$-G6P) $\beta$-D-Glucose 6-phosphate
- (6-PGDL) D-Glucono-1,5-lactone 6-phosphate
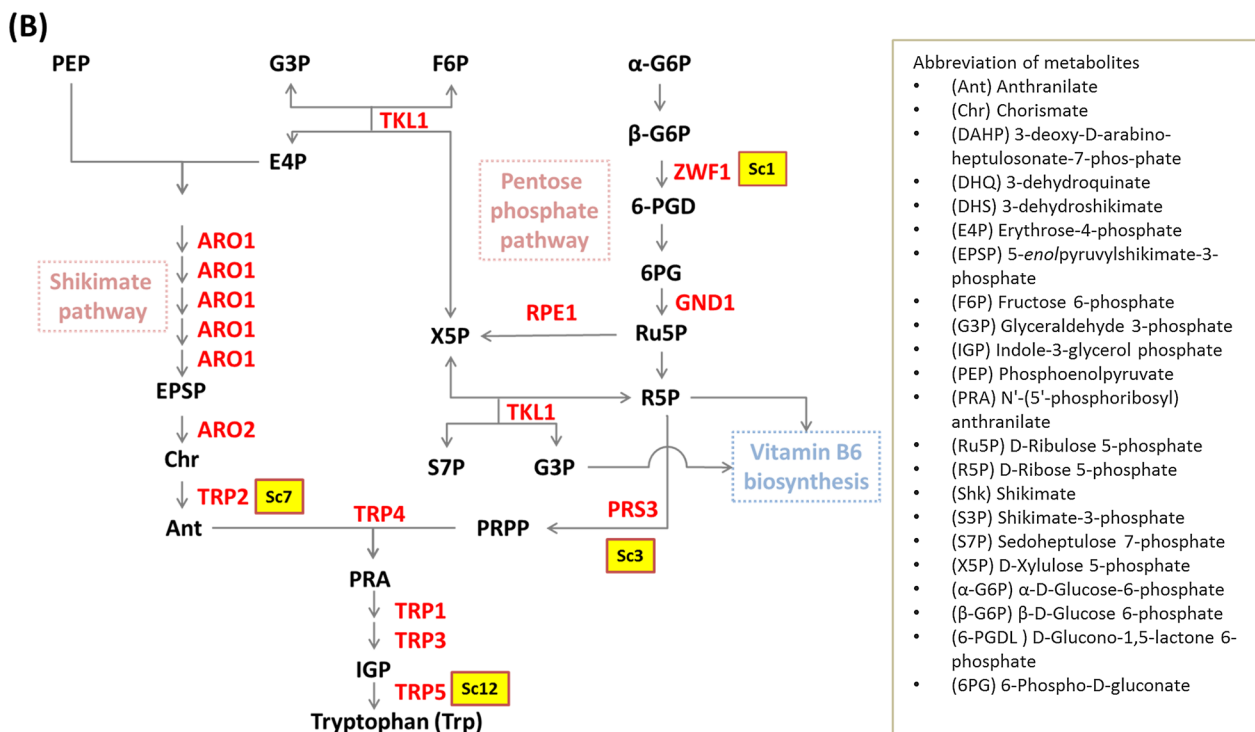- (6PG) 6-Phospho-D-gluconate

**Fig. 1** *S. cerevisiae* gene knock-out library screening for genes and pathways related to isobutanol tolerance. **a** Enrichment analysis was done by the online tool DAVID and the top 3 enriched pathways are listed here, including the tryptophan biosynthesis pathway (Annotation cluster 1), the ubiquitination pathway (Annotation cluster 2), and the pentose phosphate pathway (Annotation cluster 3). **b** Enriched biological functions including tryptophan biosynthesis and the pentose phosphate pathway. The genes related to isobutanol tolerance are labeled in red color

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tolerance. Those genes included glucose-6-phosphate dehydrogenase (*ZWF1*), phosphogluconate dehydrogenase (*GND1*), ribose phosphate diphosphokinase subunit (*PRS3*), ribulose-phosphate 3-epimerase (*RPE1*), and transketolase (*TKL1*) were identified. Interestingly, PPP is the main pathway to synthesize phosphoribosyl pyrophosphate (PRPP) and erythrose 4-phosphate (E4P), which are two important precursors for the shikimate pathway and tryptophan synthesis (Fig. 1b). These observations indicate that tryptophan or related metabolites may significantly contribute to isobutanol tolerance in yeast. It has been shown that tryptophan biosynthesis also responds to ethanol stress, while either overexpression or supplement of tryptophan could increase ethanol tolerance. Cellular membrane is a predominant target of ethanol, which decreases membrane integrity [13, 14]. Isobutanol is more hydrophobic than ethanol and is more toxic to cells by disrupting cell membrane. Stabilization of membrane has been found to protect yeast from isobutanol [15]. Tryptophan has been suggested to play a role in membrane stress response and to protect membrane from a common detergent sodium dodecyl sulfate (SDS) [16].

### Recovering and improving isobutanol tolerance by exogenous tryptophan

To confirm the importance of tryptophan in isobutanol tolerance, the mutant strain Sc7 (ΔTRP2, anthranilate synthase deletion) and Sc12 (ΔTRP5, tryptophan synthase deletion) were used to conduct functional tests. TRP2 is a pivotal enzyme converting chorismate to anthranilate for the tryptophan biosynthesis pathway, and TRP5 is the final step to the tryptophan biosynthesis pathway. Growth assay was applied to different isobutanol concentrations. At the concentration of 1% and 1.5%, the mutant strains had a lower cell density than the wild type (Fig. 2a). This indicates that the defect of tryptophan mutants caused cells sensitive to isobutanol. A tryptophan supplementation assay was then introduced to examine whether the defect can be recovered or not. The result showed that mutant strains could be recovered by adding tryptophan in a dose-dependent manner (Fig. 2b). This also suggested that cells may become more sensitive to isobutanol when the concentration of tryptophan becomes low. Although the assay used normal medium to keep basic needs for cell's living, the amount of tryptophan in medium is not enough for cells to tolerate

isobutanol. We found that at least 100 μg/ml exogenous tryptophan was needed to recover the growth rate of the defective strain at its wild-type level. Thus, 200 μg/ml was applied in subsequent experiments. In addition, the wild type showed a slightly increased tolerance with tryptophan added in the medium. Therefore, the wild type was tested again to see whether tryptophan could improve the tolerance or not. Different concentrations of isobutanol treatment on the wild type were supplemented with and without tryptophan. In 1.5% isobutanol, the wild type fed with tryptophan had a higher survival rate compared to the sample without tryptophan supplement (Fig. 2c). Adding tryptophan to medium has not provided much improvement in the wild type, which may be because it still had a functional tryptophan pathway. In order to eliminate the nutritional growth effect from tryptophan metabolism, all strains were examined in conditions either with or without tryptophan addition. The result showed almost the same growth rate (Additional file 1: Figure S1). The above results indicated that tryptophan contributed to the increased tolerance of yeast to isobutanol.

### Tryptophan could recover isobutanol tolerance of defective PPP

The pentose phosphate pathway (PPP) is an essential pathway to provide tryptophan precursors E4P and PRPP. In initial tryptophan biosynthesis, phosphoenolpyruvate (PEP) derived from glycolysis is condensed with E4P and enters into the shikimate pathway to form chorismate. Chorismate is then converted into anthranilate, which then condenses with PRPP for the tryptophan biosynthesis pathway. PPP plays a crucial role in tryptophan biosynthesis and we found that a defect in PPP makes yeast sensitive to isobutanol. The mutant strains Sc1 (ΔZWF1, glucose-6-phosphate 1-dehydrogenase deletion) and Sc3 (ΔPRS3, ribose-phosphate pyrophosphokinase deletion) were selected for further characterization. ZWF1 is the first rate-limiting step to introduce the metabolic flux into PPP, and PRS3 is a key enzyme to produce PRPP for tryptophan biosynthesis. Sc1 and Sc3 grew more slowly under isobutanol treatment than the wild type, and both could be recovered by adding tryptophan (Fig. 3a; Additional file 1: Figure S2). However, when we checked the recovery time, Sc1/Sc3 recovered more slowly than Sc7/Sc12 (Fig. 3b, c). This suggests that tryptophan can rescue tryptophan biosynthesis defect more directly than

(See figure on next page.)
**Fig. 2** Growth assay related to the tryptophan biosynthesis pathway and isobutanol tolerance. **a** Growth assay (24 h) of the tryptophan biosynthesis pathway mutant strains Sc7and Sc12, and wild type (WT) in different isobutanol concentrations. Sc7 and Sc12 were more susceptible to isobutanol compared to WT. **b** The growth rate of defective mutant strains Sc7 and Sc12 can be recovered by adding exogenous tryptophan (24 h). **c** Wild type showed improvement in isobutanol tolerance after external tryptophan (200 μg/ml) was supplemented. The data represent the mean ± SD (*n* = 3) (48 h)

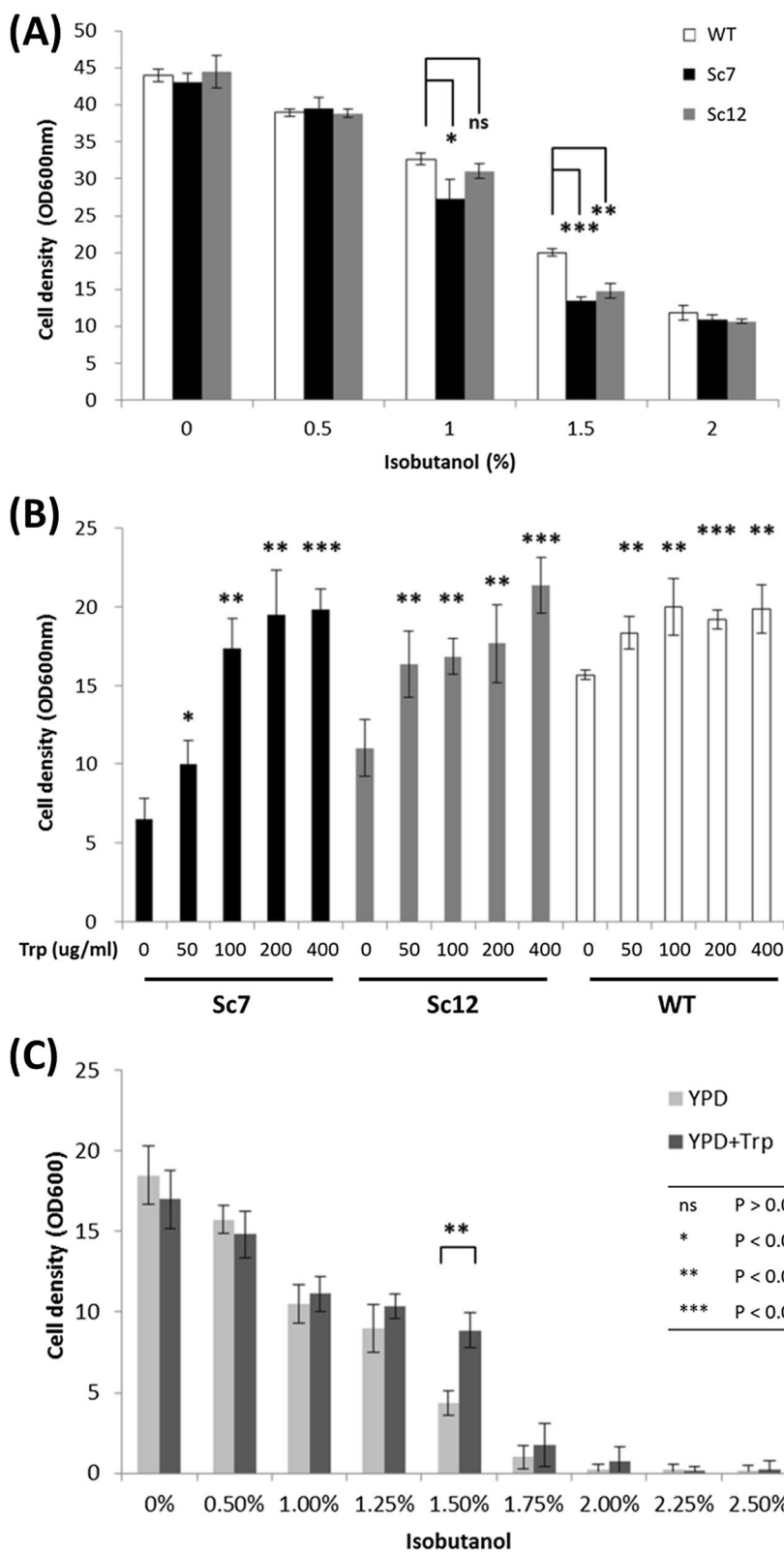Liu *et al. Biotechnol Biofuels*     (2021) 14:200

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**Fig. 2** (See legend on previous page.)

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PPP defect. Thus, the PPP pathway seems to be involved in isobutanol tolerance through the tryptophan pathway. Sc3 showed most severe growth defect compared to the other 3 mutant strains, but it could be recovered by adding tryptophan. The result suggests that PRPP for tryptophan biosynthesis is important for isobutanol tolerance (Fig. 3d). For this reason, we subsequently focused on profiling the genetic responses, and surveyed the relationships between isobutanol stress and tryptophan through transcriptomic analysis by next-generation sequencing.

## Transcriptional responses to isobutanol stress and exogenous tryptophan

To evaluate the cellular response to isobutanol and tryptophan, transcriptomic analysis was conducted to characterize the transcriptome profile and pathway regulation. The wild type and mutant strain Sc12 were subject to 4 different conditions for 24 h: no treatment, tryptophan treatment, isobutanol treatment, and isobutanol plus tryptophan. Each sample was obtained from three replicates, growth conditions were checked before RNA harvesting (Additional file 1: Figure S3), and knockout of *TRP5* was verified by polymerase chain reaction (PCR) and RNA sequencing (Additional file 1: Figure S4a, b). The sequencing reads were mapped to the reference genome (UCSC sacCer3) by Tophat (Additional file 1: Table S2), and the FPKM (fragments per kilobase of transcript per million) was calculated by Cufflinks. To characterize gene expression patterns, heatmaps and principal component analysis (PCA) plots were obtained in terms of FPKM values (Fig. 4a, b). Based on the PCA plots and the heatmaps, the Sc12 strain treated with isobutanol was very different from the other samples. When external tryptophan was added to Sc12 with isobutanol, the gene expression pattern shifted closer to the normal condition, suggesting that tryptophan mitigated the effects of isobutanol (Sc12 + IB vs. Sc12 + IB + Trp). On the other hand, the wild type showed only a slight change in expression pattern when tryptophan was added to the medium that contained isobutanol (WT + IB vs. WT + IB + Trp). Furthermore, both the wild type and the mutant strain did not show much change in the transcriptome when supplemented with tryptophan (WT vs. WT + Trp/Sc12 vs. Sc12 + Trp). The result suggests that tryptophan did not significantly alter the growth of WT and Sc12, but specifically

mitigated the effect of isobutanol on Sc12. The overall transcriptome pattern seems to reflect the growth assay, and was reliable for further differential expression analysis. Table 1 shows the number of differentially expressed genes (DEGs) for each pair and the cutoff threshold was set to $q$ value = 0.05 and fold-change = ± 2. The DEG numbers of WT versus WT + Trp (7) and Sc12 versus Sc12 + Trp (145) were small, implying that exogenous tryptophan did not have much effect on growth. In addition, when we compared WT + IB and WT + IB + Trp, only 14 genes showed significant changes in expression level, suggesting that external tryptophan has no significant effect on the gene expression of the wild type. In order to know the biological functions in which the DEGs participated, enrichment analysis of all paired groups was done. The enriched pathways mainly include biosynthesis of amino acids, the pentose phosphate pathway, tryptophan metabolism, the nicotinamide adenine dinucleotide (NAD) biosynthetic process, fatty acid metabolism, thiamine (Vitamin B$_1$) metabolism, and vitamin B$_6$ metabolism. The gene expression level of each enriched pathway was subject to cluster analysis to reveal their relationships.

### Biosynthesis and transportation of amino acids responding to isobutanol stress in a strain with a defective tryptophan biosynthesis pathway

Biosynthesis of amino acids seems to play an important role in mitigating the effect of isobutanol stress when the tryptophan biosynthesis pathway is defective. The cluster analysis showed that many genes participating in amino acid biosynthesis were highly expressed when wild type was treated with isobutanol. Compared to wild type, the mutant strain Sc12 showed stronger expression pattern when treated with isobutanol, but were then down-regulated by tryptophan supplementation (Fig. 5a). The up-regulated biosynthesis pathways included Ala, Gly, Ser, Pro, His, Phe, Tyr, Trp, Val, Leu, Ile, Cys, Met, Thr, Asp, Asn, Lys, Glu, Gln, and Arg, suggesting that cells require a broad range of amino acids to tolerate isobutanol stress when the tryptophan biosynthesis pathway is defective. In addition, about half of amino acid transporters showed high expression under isobutanol stress. Other transporters were up-regulated in Sc12 under isobutanol stress and down-regulated after adding tryptophan, in which almost all-natural amino acid transporters directed amino acids from extracellular to cytosol (Fig. 5b). These

**Fig. 3** Growth assay related to the pentose phosphate pathway and isobutanol tolerance. **a** Growth assay was applied to two pentose phosphate pathway (PPP) defective strains (Sc1 and Sc3). The two mutant strains showed more sensitive to isobutanol (1%) compared to the wild type. **b**, **c** Two PPP-defective strains (Sc1 and Sc3) and two tryptophan pathway defective strains (Sc7 and Sc12) were used in recovery assays and the two tryptophan pathway defective strains were recovered earlier **(b)** than the two PPP-defective strains **(c)**. **d** All defective strains were recovered at 70 h. The recovery assays was performed at 200 µg/ml tryptophan addition
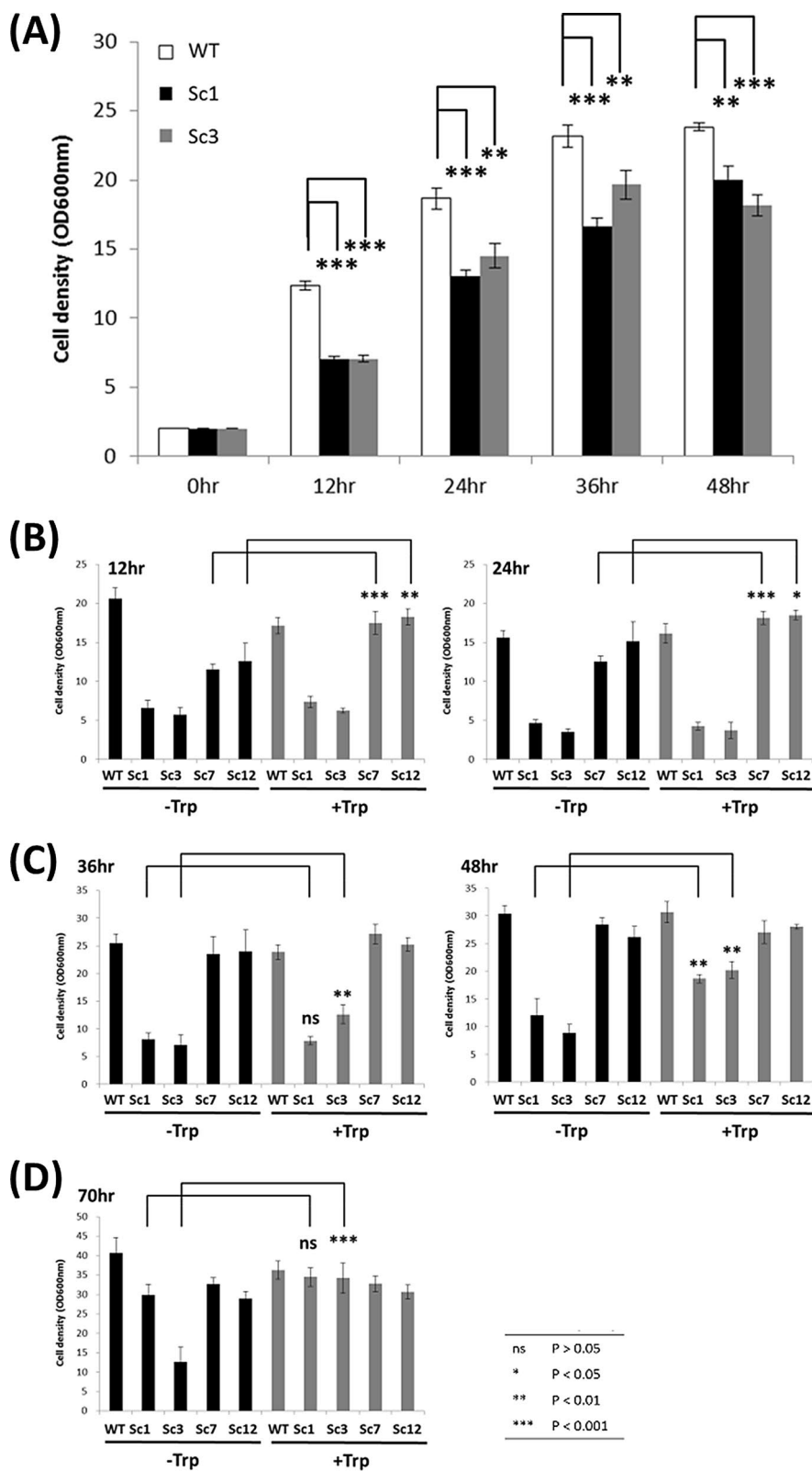
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**Fig. 3** (See legend on previous page.)

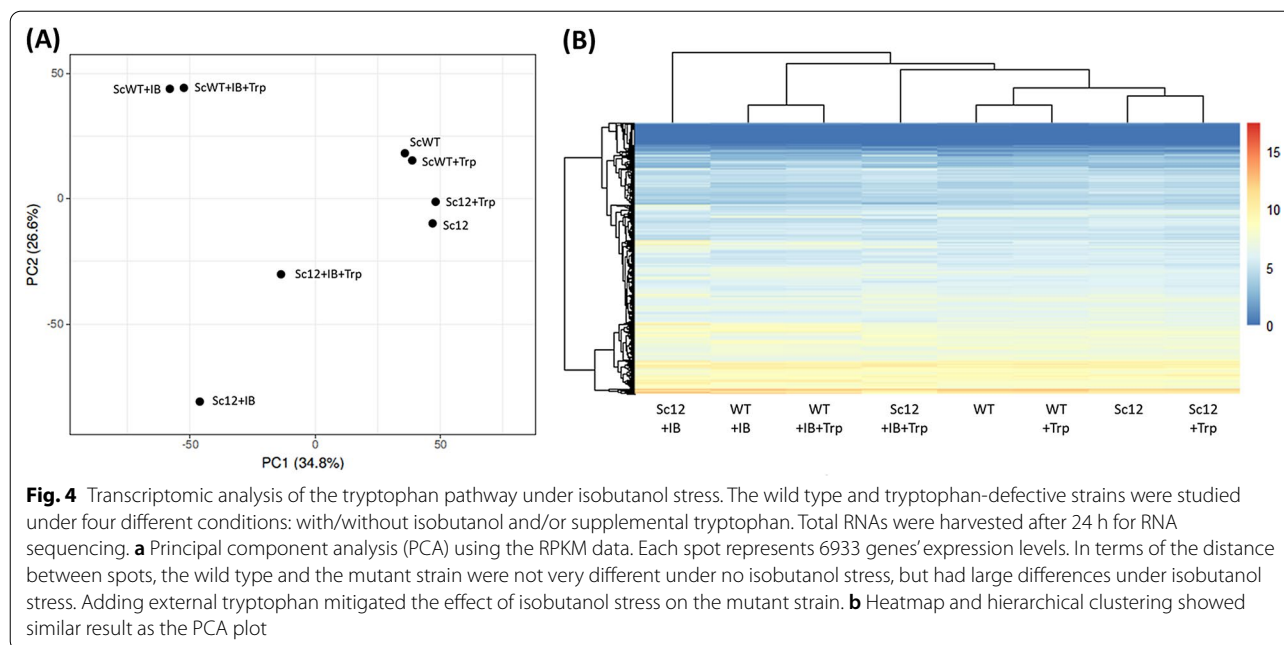Liu *et al. Biotechnol Biofuels* (2021) 14:200

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**Fig. 4** Transcriptomic analysis of the tryptophan pathway under isobutanol stress. The wild type and tryptophan-defective strains were studied under four different conditions: with/without isobutanol and/or supplemental tryptophan. Total RNAs were harvested after 24 h for RNA sequencing. **a** Principal component analysis (PCA) using the RPKM data. Each spot represents 6933 genes' expression levels. In terms of the distance between spots, the wild type and the mutant strain were not very different under no isobutanol stress, but had large differences under isobutanol stress. Adding external tryptophan mitigated the effect of isobutanol stress on the mutant strain. **b** Heatmap and hierarchical clustering showed similar result as the PCA plot

**Table 1** Number of differentially expressed genes (DEGs) between each sample pair

|  | WT | WT + Trp | WT + IB | WT + IB + Trp | Sc12 | Sc12 + Trp | Sc12 + IB | Sc12 + IB + Trp |
|---|---|---|---|---|---|---|---|---|
| WT | – |  |  |  | 408 |  |  |  |
| WT + Trp | 7 | – |  |  |  | 290 |  |  |
| WT + IB | 1374 |  | – |  |  |  | 1628 |  |
| WT + IB + Trp |  | 1313 | 14 | – |  |  |  | 1379 |
| Sc12 |  |  |  |  | – |  |  |  |
| Sc12 + Trp |  |  |  |  | 145 | – |  |  |
| Sc12 + IB |  |  |  |  | 1768 |  | – |  |
| Sc12 + IB + Trp |  |  |  |  |  | 1361 | 1506 | – |

The cutoff threshold was set up as *q* value = 0.05 and fold-change = ± 2

*Trp* tryptophan; *IB* isobutanol

observations indicate that the cells try not only to synthesize but also to import amino acids, and tryptophan seems to play a key role in this process. Meanwhile, transketolase *TKL1* (YPR074C) and transaldolase *TAL1* (YLR354C) were slightly over-expressed to activate the

pentose phosphate pathway (PPP) non-oxidative phase, trying to awake tryptophan biosynthesis upstream in the defective strain, and were down-regulated after adding tryptophan. In addition, some PPP-related genes were highly expressed under isobutanol stress in the wild
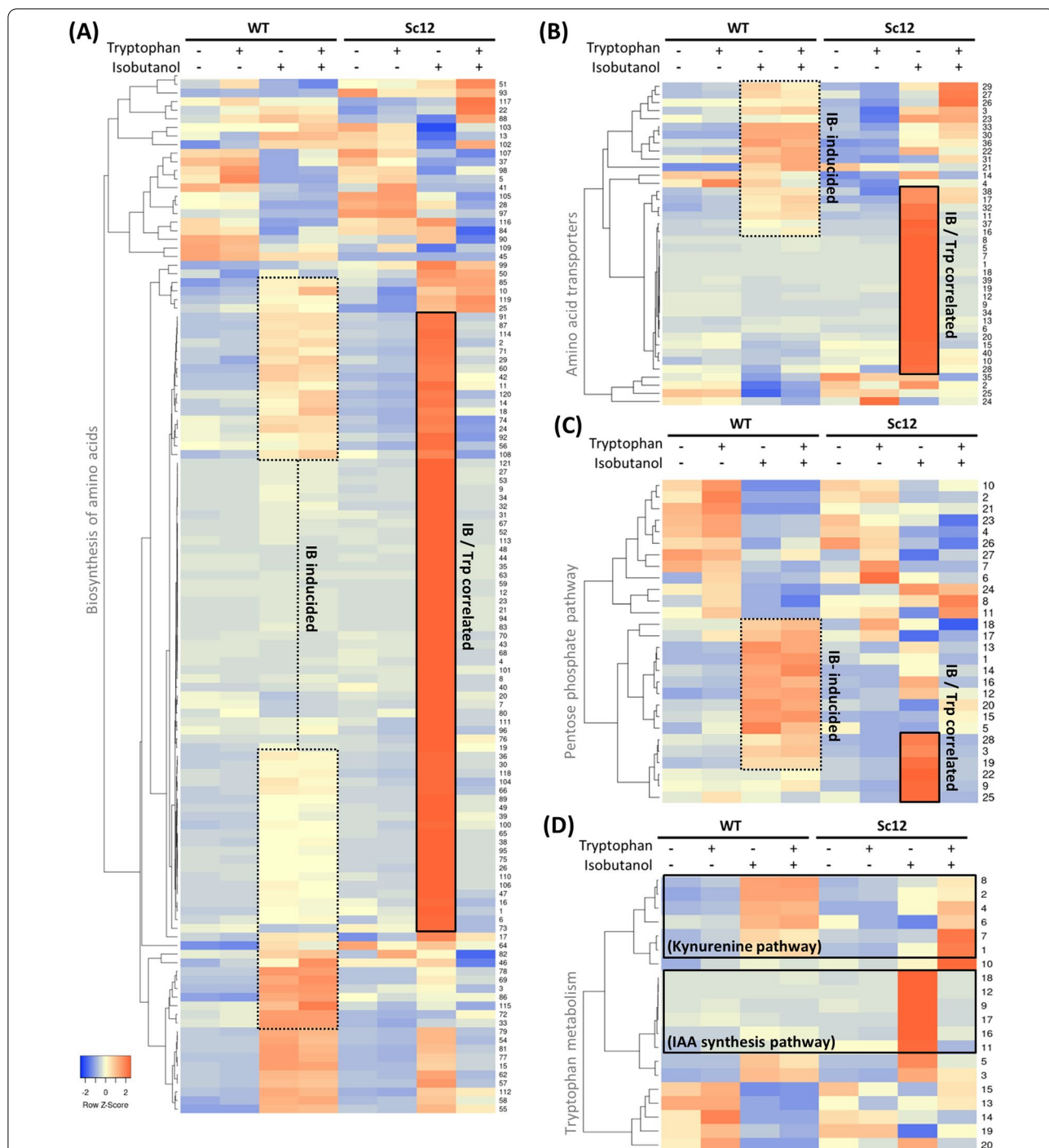
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**Fig. 5** Clustering analysis of enriched cell functions related to isobutanol stress and tryptophan supplementation. The expression changes between each sample pair were subject to enrichment analysis. The enriched cellular functions were then clustered to exhibit the gene expression pattern differences between the high and low expression levels in each sample, which were represented by red and blue color, respectively. The enriched cell functions include: **a** biosynthesis of amino acids, **b** amino acid transporters, **c** the pentose phosphate pathway, **d** tryptophan metabolism, **e** nicotinate and nicotinamide metabolism, **f** vitamin B$_6$ metabolism, **g** thiamine (vitamin B$_1$) metabolism, and **h** fatty acid metabolism. The rectangle emphasizes the gene clusters related to isobutanol stress and tryptophan. The number to the right-hand side represents every single gene applied to each heatmap. Gene names of each heatmap are listed in Additional file 1: Table S3

**Fig. 5** continued
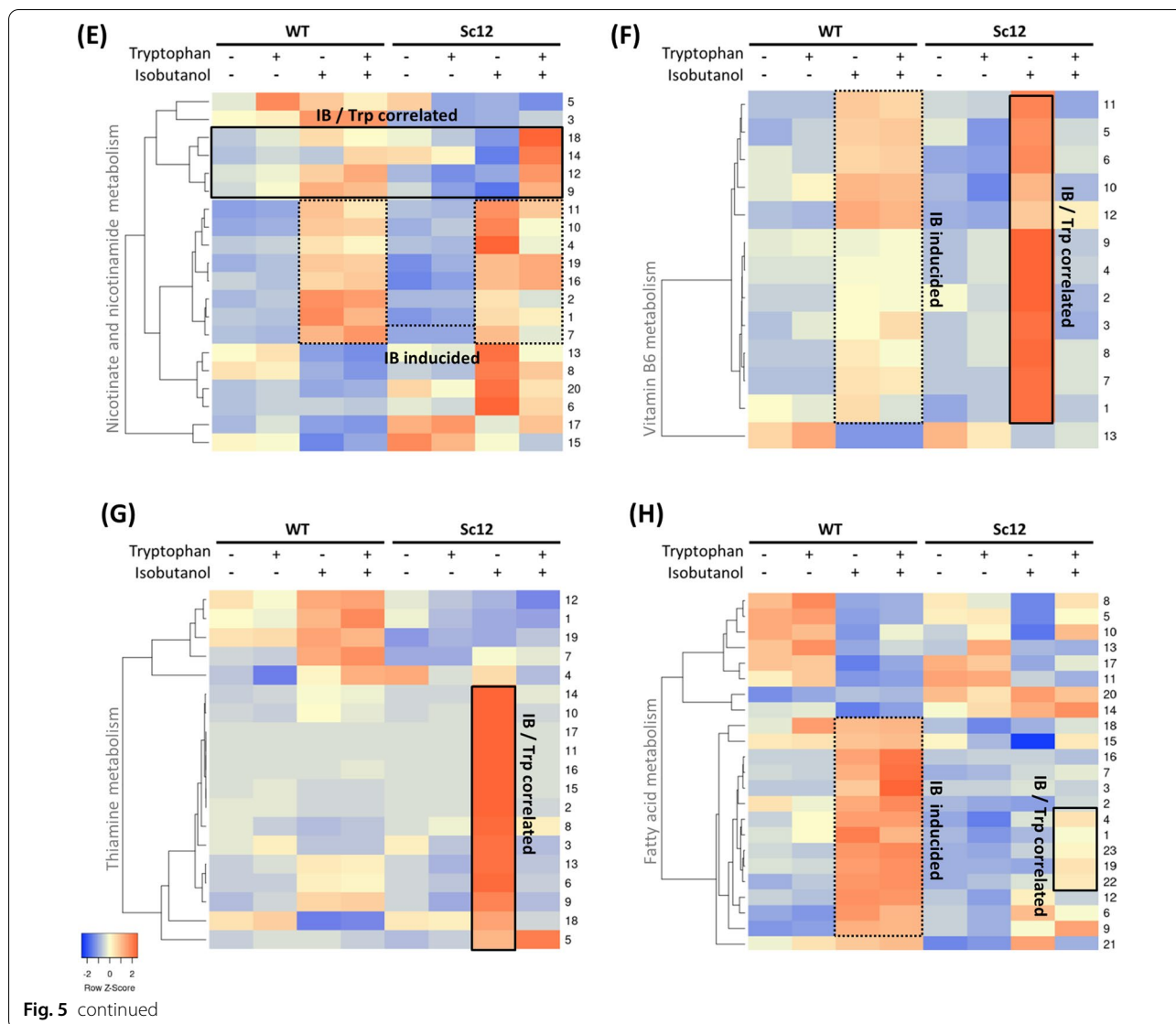
type, but down-regulated in the mutant strain (Fig. 5c). Those genes included Sol2p (*SOL2*), 6-phosphoglucono-lactonase 3 (*SOL3*), phosphogluconate dehydrogenase (*GND1*), ribulose-phosphate 3-epimerase (*PRE1*), ribose-phosphate pyrophosphokinase 1 (*PRS1*), ribose-phosphate pyrophosphokinase 3 (*PRS3*), ribose-phosphate pyrophosphokinase 4 (*PRS4*), and phosphoglucomutase (*PGM1*). The high expression of *TKL1*, *GND1*, *RPE1*, and *PRS3* under isobutnaol stress confirmed the result in knock-out strains screening, in which deletion of any of these genes was susceptible to isobutnal. This supports the view that the PPP is related to the tryptophan pathway for isobutanol tolerance.

### Tryptophan downstream metabolism also plays an important role in yeast's tolerance to isobutanol

Tryptophan metabolism involves processes to convert tryptophan to many downstream bioactive molecules such as melatonin, kynurenine, indole-3-acetic acid (IAA), and nicotinamide adenine dinucleotide (NAD$^+$). Cluster analysis of genes involved in tryptophan metabolism resulted in two groups, one enriched in the kynurenine pathway and the other enriched in the IAA biosynthesis pathway (Fig. 5d). Genes in the kynurenine pathway, such as tryptophan 2,3-dioxygenase (*BNA2*), formylkynurenine formamidase (*BNA7*), and kynureninase (*BNA5*), were highly expressed when WT was treated with isobutanol, but were down-regulated when the mutant strain Sc12 was treated with isobutanol. After

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adding tryptophan to Sc12, genes in the kynurenine pathway were up-regulated, implying that the activation of the kynurenine pathway requires tryptophan. The kynurenine pathway of tryptophan metabolism exhibits antioxidant property to eliminate oxidative stress, especially using the enzyme indoleamine 2,3-dioxygenase (IDO) [17, 18]. IDO, named BNA2 in *S. cerevesia*, was also highly expressed when WT was treated with isobutanol but down-regulated in Sc12 when it was treated with isobutanol, and it could be up-regulated by adding tryptophan (Additional file 1: Figure S5). In addition, the kynurenine pathway is the only de novo biosynthesis pathway of nicotinamide adenine dinucleotide (NAD). $NAD^+$ can be phosphorylated to $NADP^+$ by $NAD^+$ kinase, which can be reduced to NADPH and NADH, respectively. NAD(H) is important in regulating cellular energy metabolism, and NADP(H) is mainly involved in redox balance and biosynthesis of fatty acids and nucleic acids [19]. In the cluster analysis of nicotinate and nicotinamide metabolism, a large proportion of genes were up-regulated when the cell was treated with isobutanol. In Sc12, some of the genes did not respond to isobutanol and were reactivated when adding tryptophan. These genes, including nicotinic acid mononucleotide adenylyltransferase (*NMA2*) and glutamine-dependent NAD synthetase (*QNS1*), could downregulate $NAD^+$ production when Sc12 was under isobutanol stress (Fig. 5e), and the expression pattern was similar to the genes in the kynurenine pathway, implying that cells rely on tryptophan to synthesize $NAD^+$ through the kynurenine pathway. Thus, the function of $NAD^+$, such as redox balance regulation for fatty acid synthesis and anti-oxidative ability, may contribute to isobutanol tolerance.

In the IAA biosynthesis pathway, genes were up-regulated only when the mutant strain was under isobutanol stress, and then returned to low-level expression after tryptophan addition (Fig. 5d). This is similar to the expression pattern of amino acid biosynthesis and transport. IAA is a common natural compound in plants and microbes, and regulates plant-microbe interaction. IAA can stimulate plant cell elongation, division, and differentiation. Moreover, exogenous IAA can regulate adhesion and filamentation in *S. cerevisiae* by inducing morphological genes [20, 21]. This is consistent with the finding that filamentous growth could increase yeast isobutanol tolerance [22]. However, we did not observe obvious morphological change in both WT and Sc12 when grown under isobutanol. Although up-regulation of the IAA pathway seems to convert upstream tryptophan into downstream IAA, the occurrence of this phenomenon in the defective tryptophan biosynthesis pathway strain makes it difficult to explain. Although there is evidence that IAA has a tryptophan-independent biosynthesis pathway, which

branches from either indole or indole-3-glycerol phosphate, the specific enzymes in *S. cerevisiae* still have not been identified [23]. The actual role of IAA and its regulation of biosynthesis pathway under isobutanol stress remain to be investigated.

### Vitamins $B_6$ and $B_1$ support isobutanol tolerance

Induction of vitamin $B_6$ and vitamin $B_1$ (thiamine) metabolic genes were observed under isobutanol stress. Vitamin $B_6$ biosynthesis genes were up-regulated when the WT was treated with isobutanol. Moreover, similar to amino acid biosynthesis and tryptophan metabolism, both vitamin $B_6$ and $B_1$ genes showed a high expression level when the mutant strain Sc12 was under isobutanol stress, indicating that vitamin $B_6$ and $B_1$ genes respond to isobutanol stress and are related to tryptophan (Fig. 5f, g). Vitamin $B_6$ is a group of similar biological active compounds including pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM), and their phosphorylated derivatives pyridoxine 5′-phosphate (PNP), pyridoxamine 5′-phosphate (PMP), and pyridoxal 5′-phosphate (PLP). PLP is the active form of vitamin $B_6$ and plays as a coenzyme or substrate for many bioprocesses such as amino acid biosynthesis, lipid metabolism, and thiamine biosynthesis. The precursors of PLP are converted from the PPP intermediates (glyceraldehyde 3-phosphate and ribose 5-phosphate) and glutamine. Their related enzymes, *TKL1*, *TAL1*, and *GLN1* (glutamine synthetase), exhibited similar gene expression patterns to vitamin $B_6$ and might facilitate the biosynthesis of vitamin $B_6$. PLP is also needed for many aminotransferases and synthases, such as tryptophan synthase (*TRP5*), and this observation may explain the requirement of amino acids when a mutant strain is under isobutanol stress. Furthermore, kynureninase (*BNA5*) is a PLP-dependent enzyme to facilitate downstream tryptophan metabolism such as $NAD^+$ de novo synthesis. PLP can be further combined with histidine and thiazole moiety, derived from glycine and $NAD^+$, to form vitamin $B_1$. The major active derivative of vitamin $B_1$ is thiamine diphosphate (TDP) and serves as a cofactor for many bioprocesses such as TKL1 in PPP [24]. However, when exogenous vitamin B1 (thiamine) was added to medium in tolerance assay, there was no improvement in cell density under isobutanol stress (Additional file 1: Figure S6). This may be because, as a cofactor, vitamin B1 may not work alone to rescue the tryptophan-defective strain (Sc12). This issue requires further study.

To summarize, the enriched pathways described above seem to coordinate with each other when a cell is under isobutanol stress. Yeast tries to optimize the expression levels of genes participating in the biosynthesis or import of amino acids under conditions such as nitrogen

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starvation. The tryptophan metabolism pathway is activated and induces IAA biosynthesis, the kynurenine pathway, and nicotinamide metabolism. Nicotinamide metabolism may provide redox pool ($NAD^+/NADP^+$) and the pentose phosphate pathway (PPP) may support anti-oxidative ability. The kynurenine pathway also plays an important role against free oxidative stress. PPP, $NAD^+$, histidine and glycine provide raw materials for the synthesis of vitamins $B_6$ and $B_1$, which can then enhance amino acid biosynthesis, tryptophan metabolism, and PPP. Fatty acid metabolism genes are also up-regulated to confront isobutanol in the WT, and those genes mainly participate in fatty acid biosynthesis. This implies that fatty acid biosynthesis may help tolerate solvent stress, such as strengthening the cell membrane. In addition, some genes were down-regulated in the mutant strain and could be rescued by tryptophan, especially acetyl-coa carboxylase (*ACC1*), which is a rate-limiting step for the biosynthesis of fatty acids (Fig. 5h). The expression patterns of fatty acid synthesis genes are similar to those in the kynurenine pathway and $NAD^+$ de novo synthesis. Accordingly, tryptophan and tryptophan metabolism seems to play a role in coordinating fatty acid biosynthesis. However, the relationship between tryptophan and fatty acid synthesis is not yet clear in yeast. The increased NAD pool from tryptophan metabolism may be a reason that it provides energy for fatty acid biosynthesis for emergency. Based on our transcriptomic analysis, many biological networks seem to work together to build a defense wall to protect cells from isobutanol stress.

## Discussion

A high-throughput platform was built in this study to identify isobutanol-susceptible mutants through their colony size. A total of 5006 single-gene deletion strains were tested and 161 gene-deletions exhibited reduced growth. To understand what biological functions may be involved in isobutanol tolerance, functional enrichment analysis was applied to these 161 genes. The result indicated that tryptophan biosynthesis, ubiquitination, and PPP were the top three enriched functions. By adding exogenous tryptophan to a tryptophan biosynthesis or PPP-defective strain, the isobutanol tolerance can be recovered, implying that tryptophan is a key element in yeast's isobutanol tolerance. Kuroda et al. [10] have previously shown that tryptophan and PPP were involved in isobutanol tolerance. Their study revealed the key role of tryptophan in PPP/GLN3 and nitrogen starvation response while we provided insights into the tryptophan pathway. Furthermore, we revealed the potential relationship between tryptophan and nitrogen starvation response. Our results support the finding of Kuroda et al.

[10], and suggest the possible network behind the "isobutanol stress-induced" nitrogen starvation.

In order to understand the genetic basis of cellular functions participating in the stress tolerance, transcriptome analysis was performed to distinguish expression profiles between samples treated with/without isobutanol and/or tryptophan. Principal component analysis (PCA) and hierarchical clustering were employed to show that the defective tryptophan biosynthesis strain exhibited large differences in transcriptome patterns from the wild type, and the differences were reduced by exogenous tryptophan. Enrichment analysis and cluster analysis revealed that amino acid biosynthesis and transportation were highly expressed under isobutanol stress. The results showed a higher level of amino acid biosynthesis in the tryptophan biosynthesis-defective strain than the wild type. In contrast, the high expression level was reduced by adding tryptophan. These observations suggest that the activation of amino acid biosynthesis is closely related to the defect in tryptophan biosynthesis. In addition, a large number of amino acid transporters were also up-regulated by isobutanol when tryptophan biosynthesis was defective. These results point to the emergency demand of amino acids, similar to the hypothesis of nitrogen starvation response induced by isobutanol proposed by Kuroda et al. [10]. Their transcriptomic analyses revealed that isobutanol induces a nitrogen starvation response via *GLN3* [10]. In our transcriptomic data, the tryptophan biosynthesis mutant and its isobutanol-treated sample had increased *GLN3* expression that could be reduced by adding external tryptophan (Additional file 1: Figure S7). This implies that the *GLN3* expression level is affected by tryptophan under isobutanol stress. Furthermore, it has been shown that the level of glutamine is a key indicator of nitrogen starvation, which can activate the transcription factor *GLN3* [25]. In both *S. cerevisiae* and *E. coli*, glutamine level drastically dropped under nitrogen starvation. Under such condition, synthesis of the aromatic amino acids including phenylalanine, tyrosine, and tryptophan can follow a common pathway upstream to the key intermediate chorismate, which contains no nitrogen. Chorismate could then acquire nitrogen from glutamine to continue the tryptophan pathway [26]. In our transcriptome data, the expression levels of glutamine synthetase (*GLN1*) and vacuolar glutamine transporter (*Avt7*) were up-regulated under isobutanol stress to improve glutamine synthesize and homeostasis. The glutamine transporters, glutamine permease (*GNP1*) and dicarboxylic amino acid permease (*DIP5*), located on plasma membrane were all up-regulated when tryptophan biosynthesis-defective strains confront isobutanol stress, which may support the demand of glutamine (Additional file 1:

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Figure S8). These lines of evidence suggest an important role of glutamine in "isobutanol stress-induced" nitrogen starvation, in particular when cells consume more tryptophan under isobutanol stress. Furthermore, in Kuroda et al. [10], the *gln3Δ* strain showed lower intracellular glutamine concentration compared to the wild type regardless of the presence or absence of isobutanol. This suggests that downstream genes of *GLN3* may upregulate the ratio and concentration of glutamine/glutamate. When under isobutanol stress, *GLN3* may try to satisfy the emergent demand of glutamine.

Hence, we postulate that the tryptophan demand increased in response to isobutanol stress, triggering the need for an additional nitrogen donor, in particular glutamine. Meanwhile, the decreasing glutamine level may activate the transcriptional regulator *GLN3* for further nitrogen starvation response. The difference between "true" nitrogen starvation and "isobutanol stress-induced" nitrogen starvation is that under true nitrogen starvation there is no nitrogen supply from glutamine whereas in the "isobutanol stress-induced" nitrogen starvation cells can take advantage of chorismate and acquire nitrogen from glutamine. The expression profile of *GLN3* in Additional file 1: Figure S7 supports our hypothesis and largely explains why the expression level of *GLN3* did not change when the wild type was treated with isobutanol. In addition, when a cell has a defective tryptophan pathway, *GLN3* and the glutamine-related genes (*GLN1*, *DIP5*, and *GNP1*) were highly up-regulated (Additional file 1: Figures S7, 8) and the growth defect became more severe than the wild type (Additional file 1: Figure S3). After the addition of exogenous tryptophan, the expression levels of *GNL3* and the glutamine-related genes were decreased and the growth defect was recovered (Additional file 1: Figure S3). These results were consistent with the previous finding that the *GLN3* knock-out strain showed better tolerance to isobutanol [10].

On the other hand, ubiquitination was enriched in our knockout screening analysis, which is consistent with the result of González-Ramos et al. [12], indicating that protein degradation and turnover have been activated during the butanol challenge in *S. cerevisiae*. The biologically active $B_6$ vitamin PLP is the coenzyme involved in protein synthesis, degradation, and amino acid interconversion. Indeed, the gene expression patterns of amino acid synthesis and their transporters (Fig. 5a, b) and vitamin $B_6$ synthesis (Fig. 5f) appeared highly allied. Thus, we suggest that protein turnover may facilitate isobutanol tolerance in yeast by providing free amino acids as a response to nitrogen starvation.

It is interesting that the exogenous tryptophan rescued the PPP defect more slowly than the tryptophan pathway defect in yeast. Many biochemical reactions rely on PPP as a precursor supplier. PPP is not only an important upstream pathway of tryptophan biosynthesis, but also is needed for the cofactors NAD/NADP, nucleotides, and some other amino acids biosynthesis. In addition, PPP also provides glyceraldehyde 3-phosphate and R5P as precursors for vitamin $B_6$ synthesis, while vitamin $B_6$ is involved in the reactions of tryptophan synthase (*TRP5*) and kynureninase (*BNA5*). Thus, PPP plays multiple roles in both upstream tryptophan biosynthesis and downstream tryptophan metabolism. We speculate that although tryptophan supplementation does not immediately rescue the defect in PPP, exogenous tryptophan may reduce the consumption of PPP intermediates used for isobutanol tolerance. The conservation of more PPP intermediates can be used for other biosynthesis that is needed for cell survival. As a result, cell growth of the PPP-defective strain is gradually recovered by tryptophan supplementation. This could explain why the tryptophan rescue on PPP mutants occurred much later than that in tryptophan mutants. Our view is supported by Kuroda et al. [10] report that deletion of PPP genes *GND1* or *ZWF1* causes hypersensitivity to isobutanol. In addition, PPP gene expression pattern was similar to both tryptophan and vitamin $B_6/B_1$ biosynthesis, suggesting that PPP might support the synthesis of precursors for tryptophan and vitamin $B_6/B_1$ under isobutanol stress. Furthermore, TPP-dependent enzyme transketolase *TKL1* (YPR074C) was over-expressed in isobutanol-treated Sc12, which is also consistent with the finding of vitamin $B_1$ activation. Oxidative and osmotic stresses increased the production of numerous vitamin $B_1$ biosynthesis enzymes in yeast, indicating a protective role of vitamin $B_1$ against adverse environmental conditions. Mutations of vitamin $B_1$ synthesis genes increased the sensitivity to oxidative stress, suggesting that thiamine metabolism can partly compensate damages of yeast general defense systems [27].

The tryptophan mutant strain seemed to have reduced gene expression related to the kynurenine pathway. The kynurenine pathway in tryptophan metabolism is the only de novo biosynthesis pathway of nicotinamide adenine dinucleotide (NAD). This suggests that tryptophan may activate the kynurenine pathway and nicotinamide metabolism for isobutanol tolerance. Over-expressing the first biosynthesis of NAD + (kynurenine) pathway gene *BNA2* skews glycolytic flux from lipid droplets towards the aromatic amino acid biosynthesis-BNA pathways. This reduces lipid droplet accumulation during aging and extends lifespan of yeast [28]. Kynurenine exhibits

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antioxidant property that eliminates oxidative stress using the enzyme indoleamine 2,3-dioxygenase (IDO). IDO is a unique enzyme that utilizes superoxide anion radical ($O_2^*$-) as both a substrate and a cofactor. The antioxidant activity may arise from $O_2^*$-scavenging by IDO and formation of the potent radical scavengers and kynurenine pathway metabolites, 3-hydroxyanthranilic acid and 3-hydroxykynurenine [18, 29]. Therefore, IDO has been proposed to be an antioxidant enzyme [30]. In addition, PPP may also provide energy to NADPH for anti-oxidative ability. This hypothesis is supported by Kuroda et al. [10] observation that the NADPH/NADP + ratio was decreased in PPP-defective strains (*zwf1Δ*). Furthermore, the isobutanol-specific sensitivity of PPP non-oxidative phase deletion (*tkl1Δ*) observed by Kuroda et al. [10] can be explained by our hypothesis that the upstream genes of tryptophan biosynthesis in PPP contribute to isobutanol tolerance. This also suggests that both of PPP and tryptophan metabolism may attenuate oxidative stress created by isobutanol.

Furthermore, the generation of NAD(P) from the kynurenine pathway is not only important in redox balance but also involved in the biosynthesis of fatty acids [31] that are needed for cell membrane formation. Fatty acid is the major source for cell membrane, so it may contribute to isobutanol tolerance. Isobutanol induced many fatty acid synthesis genes in WT, though not in the Sc12, regardless of tryptophan supplementation (Fig. 5h). These results indicate that tryptophan synthase is essential for isobutanol-induced fatty acid synthesis. Despite that adding tryptophan did not recover all down-regulated genes in the mutant strain Sc12, some key genes were rescued by exogenous tryptophan, such as *ACC1*, which catalyzes the rate-limiting step of fatty acid biosynthesis (Additional file 1: Figure S9). Thus, we postulate that tryptophan enhances cell membrane integrity via fatty acid synthesis. Tryptophan supplementation to culture and/or overexpression of the tryptophan permease gene has been found to be effective for increasing ethanol stress tolerance [32]. Tryptophan confers the *S. cerevisiae* resistance to SDS-induced cell membrane disruption. Overexpression of tryptophan permease gene *TAT2* rescues cell growth in SDS-treated Δ*mck1* cells [16], and *TAT2* confers high-pressure growth in *S. cerevisiae* [33]. Tryptophan import was enhanced upon yeast exposed to SDS, and internal concentration of tryptophan makes cells more resistant to membrane disruption caused by sodium dodecyl sulfate [16]. Our data also showed the enhancement of *TAT2* expression under isobutanol stress (Additional file 1: Figure S10). In addition, although there is no morphological change in our observation, we found that the IAA biosynthesis pathway was increased in the tryptophan-defective strain under isobutanol stress,

and IAA may be related to the filamentous phenotype. Although the literature rarely described the mechanism of tryptophan on yeast cell wall/membrane protection, the above studies implicate that tryptophan may contribute to cell wall/membrane integrity during isobutanol stress.

In summary, the defense of yeast against isobutanol stress is highly dependent on tryptophan and related networks. Under isobutanol stress, the depletion of tryptophan may acquire glutamine as the supplement of nitrogen followed by the chorismate pathway. Consequently, the lower level of glutamine alters *GLN3* expression and triggers the nitrogen starvation responses which enable the cells to manage the stress by activation of protein turnover, amino acid synthesis and import that helps replenish glutamine and tryptophan. Our transcriptomic analysis also revealed the correlated cell functions surrounding tryptophan, such as the kynureninase pathway of tryptophan metabolism and nicotinamide metabolism. These metabolisms may provide a NAD(P) pool, while PPP roles as the main resource to convert NADP to NADPH for anti-oxidative ability and fatty acid synthesis. Vitamins $B_6$ and $B_1$ synthesis genes may be up-regulated to assist these metabolic processes including amino acid biosynthesis, PPP, and lipid metabolism. Our results demonstrate the dynamics of yeast cell under isobutanol stress and tryptophan is important for its tolerance.

## Conclusions

A high-throughput colony screening method provided an efficient way to reveal the key biological functions involved in isobutanol tolerance. The growth of strains defective in either the tryptophan biosynthesis pathway or the PPP pathway could be recovered by exogenous tryptophan when they were under isobutanol stress, implying the importance of tryptophan biosynthesis in yeast's isobutanol tolerance. Based on the cluster analysis of transcriptomic data, many cell functions including amino acid biosynthesis/transport, tryptophan metabolism, vitamin $B_6$ and $B_1$ biosynthesis, and fatty acid metabolism were found to coordinate with each other under isobutanol stress. Based on this and previous studies, we suggest that the *GLN3*-induced nitrogen starvation may be due to depletion of tryptophan and its nitrogen donor glutamine. Our study highlights the central role of tryptophan in yeast's tolerance to isobutanol and offers new clues for engineering yeast with higher isobutanol tolerance.

## Methods

### Growth assay

*Saccharomyces cerevisiae* BY4741 was used in this study. All strains under study were grown in the YPD medium

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composed of yeast extract 10 g/L, peptone 20 g/L, and glucose 20 g/L. G418 (200 μg/mL) was added into YPD to maintain the Yeast Knockout Collection. Cells were thawed from a − 80℃ stock and were precultured in fresh medium for 24 h before assay. The isobutanol tolerance assay was conducted in the YPD medium with/without tryptophan (200 μg/ml). After 24 h, the cell density was measured by optical density at a wavelength of 600 nm using disposable cuvettes (10 mm). In recovery assay, cells were cultured in YPD medium with 1% isobutanol and different concentrations of tryptophan, and cell density was measured after 24 h. The wild-type tolerance assay was tested by adding 200 μg/ml tryptophan and cell density was measured after 24 h and 48 h. The recovery assay monitor at different time points was tested with/without 200 μg/ml tryptophan under 1% isobutanol stress. In colony assay, the yeast strains were inoculated in YPD liquid medium with 1% Isobutanol for 24 h, and then were serially diluted on YPD agar plates with 5 μl loading volume for 48 h incubation. The growth temperature was set at 30 ℃.

### Screening the yeast gene-deletion library

YKO MATa Strain Collection-Glycerol Stocks (Catalog #: YSC1053) was used as a gene-deletion library to screen isobutanol-sensitive mutant strains. The square plate composed of YPD agar with/without 2% isobutanol was prepared for colony array inoculation. The deletion library was precultured in 96-well plate with 200 μl medium (30 °C) to reach cell density around OD600 value of 0.8–1.0. The ROTOR HAD (Singer, USA) used 96-array pins to touch and transfer the yeast from liquid plate to solid plate. The 96-well format from liquid plate is pinned onto square solid plate four times (4-replicates), which becomes a 384 format. One square plate can accommodate 1536 colonies array. The colony size was scanned and quantified by "ScreenMill" for high-throughput image analysis [11]. We compared the colony size, from multiple replicates, between growth media with and without isobutanol, and also between each deletion strain and the wild-type strain. Growth abnormality caused by the presence of isobutanol alone (but not by the knockout gene of interest) was factored out through statistical analysis of wild-type colony sizes with or without isobutanol. Statistical analysis was then conducted by ScreenMill web-based program Statistics Visualization Engine (SV Engine). The *p* value of 0.05 was set as the threshold to output isobutanol-susceptible genes for further analysis.

### RNA sequencing

The yeast was incubated in 4 ml YPD at start $OD_{600\ nm}$ 0.5. The sample was treated with/without 1.2% Isobutanol

and/or Trp 200 μg/ml for assay in three replicates. After 24 h, cells were harvested for total RNA extraction using QIAGEN RNeasy® Mini kit (Qiagen, Germany). RNA quality was verified by Qubit™ RNA HS Assay Kit and Qubit Fluorometer (Thermo Fisher, USA). RNA Library was prepared by SuperScript™ III Reverse Transcriptase (Thermo Fisher) and TruSeq RNA LT kits (Illumina, USA) for fragment size around 350–500 bps. Sequencing was done by HiSeq 2500 with 50-bp single-end reads. Raw reads were trimmed by Trimmomatic (v0.39) and aligned to *S. cerevisiae* S288c genome (UCSC sacCer3) by TopHat. Cufflink was used to calculate FPKM values and differentially expressed genes (DEGs) were identified with the criteria of |Log2(Fold change)| > 1 and *q* value < 0.05. Enrichment analysis of GO terms and the KEGG pathway was done by the web tool "DAVID Bioinformatics Resources 6.8" (https://david.ncifcrf.gov/tools.jsp) based on DEGs [34]. The principal components analysis (PCA) plot was processed by web tool "ClustVis" (https://biit.cs.ut.ee/clustvis/) using FPKM values as the expression levels [35]. Heatmap and hierarchical clustering were made using the R package "pheatmap (version 1.0.12)" and the web tool "Heatmapper" (www.heatmapper.ca/) [36, 37]. Clustering was based on the KEGG list enriched by the DEGs in each sample.

### Abbreviations
DEGs: Differentially expressed genes; PPP: Pentose phosphate pathway; DEF1: DNA damage-responsive RNA polymerase-degradation factor; UBC8: E2 ubiquitin-conjugating protein; GRR1: SCF ubiquitin ligase complex subunit; SLX5: SUMO-targeted ubiquitin ligase complex subunit; SLX8: SUMO-targeted ubiquitin ligase complex subunit; DOC1: Anaphase promoting complex subunit; UBP13: Ubiquitin-specific protease; UBP15: Ubiquitin-specific protease; ARO1: Pentafunctional AROM protein; ARO2: Chorismate synthase; TRP2: Anthranilate synthase; TRP4: Anthranilate phosphoribosyltransferase; TRP1: Phosphoribosylanthranilate isomerase; TRP3: Indole-3-glycerol-phosphate synthase; TRP5: Tryptophan synthase; ZWF1: Glucose-6-phosphate dehydrogenase; GND1: Phosphogluconate dehydrogenase; PRS3: Ribose phosphate diphosphokinase subunit; RPE1: Ribulose-phosphate 3-epimerase; TKL1: Transketolase; PRPP: Phosphoribosyl pyrophosphate; E4P: Erythrose 4-phosphate; SDS: Sodium dodecyl sulfate; PEP: Phosphoenolpyruvate; PCR: Polymerase chain reaction; FPKM: Fragments per kilobase of transcript per million; PCA: Principal component analysis; NAD: Nicotinamide adenine dinucleotide; Trp: Tryptophan; IB: Isobutanol; His: Histidine; Phe: Phenylalanine; Tyr: Tyrosine; Trp: Tryptophan; Val: Valine; Leu: Leucine; Ilu: Isoleucine; Cys: Cysteine; Met: Methionine; Thr: Threonine; Asp: Aspartate; Asn: Asparagine; Lys: Lysine; Glu: Glutamate; Gln: Glutamine; Arg: Arginine; SOL2: Sol2p; SOL3: 6-Phosphogluconolactonase 3; GND1: Phosphogluconate dehydrogenase; PRE1: Ribulose-phosphate 3-epimerase; PRS1: Ribose-phosphate pyrophosphokinase 1; PRS3: Ribose-phosphate pyrophosphokinase 3; PRS4: Ribose-phosphate pyrophosphokinase 4; PGM1: Phosphoglucomutase; IAA: Indole-3-acetic acid; BNA2: Tryptophan 2,3-dioxygenase; BNA7: Formylkynurenine formamidase; BNA5: Kynureninase; IDO: Indoleamine 2,3-dioxygenase; NMA2: Nicotinic acid mononucleotide adenylyltransferase; QNS1: Glutamine-dependent NAD synthetase; PN: Pyridoxine; PL: Pyridoxal; PM: Pyridoxamine; PNP: Pyridoxine 5′-phosphate; PMP: Pyridoxamine 5′-phosphate; PLP: Pyridoxal 5′-phosphate; GLN1: Glutamine synthetase; Bna5: Kynureninase; TDP: Thiamine diphosphate; ACC1: Acetyl-coa carboxylase; GLN1: Glutamine synthetase; Avt7: Vacuolar

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glutamine transporter; GNP1: Glutamine permease; DIP5: Dicarboxylic amino acid permease.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13068-021-02048-z.

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**Additional file 1: Figure S1.** Growth assay of a tryptophan biosynthesis defective strain under a standard growth condition. The wild type and a tryptophan biosynthesis defective strain were tested in a standard medium with or without tryptophan addition (200 µg/ml). The growth rate shows no significantly difference between strains and between media with and without tryptophan addition. The data represent the mean $\pm$ SD ($n = 3$). **Figure S2.** Survival test of a tryptophan biosynthesis defective strain (Sc12) and a pentose phosphate pathway defective strain (Sc3). Both mutant strains Sc3 and Sc12 were sensitive to isbutanol, but could be recovered by adding tryptophan. **Figure S3.** Cell density assays of the wildtype and a tryptophan biosynthesis defective strain (Sc12) under different growth conditions. Yeasts were inoculated in 4ml medium with/without isobutanol and/or tryptophan. After 24 hours, the cell density was measured and RNA was harvested. The data represent the mean $\pm$ SD ($n = 3$). Notations: Trp, Tryptophan; IB, isobutanol. **Figure S4.** Checking the gene deletion and expression level of the mutant gene *TRP5*. **a** PCR was applied to verify the gene deletion from genome. The arrows represent the direction of 6 primers, which include A, B, C, D, kB, and kC. **b** The expression levels of the *TRP5* gene under different growth conditions were estimated from NGS data. **Figure S5.** Expression level of gene *BNA2*. *BAN2* is responsible for de novo biosynthesis of NAD+ from tryptophan via kynurenine pathway. It was highly expressed in WT and Sc12 under isobutanol. **Figure S6.** The recovery assay of wild type (WT) and tryptophan defective strain (Sc12) by supplementing of vitamin B1 (50 µg/ml) or tryptophan (200 µg/ml) under isobutanol (1%) stress. Under isobutanol stress, Sc12 can be only recovered by trptophan. Vitamin B1 did not help Sc12 to tolerate isobutanol. **Figure S7.** Expression level of gene *GLN3*. *GLN3* plays a key role in yeast's response to nitrogen starvation, including depletion of glutamine. It was upregulated when Sc12 was under isobutanol pressure; its expression level could be recovered by adding external tryptophan. **Figure S8.** Gene expression related to glutamine biosynthesis and transporter. The gene expression related to glutamine biosynthesis and transport was affected by adding tryptophan (Trp) and/or isobutanol (IB). *GLN1* was upregluated in WT when isobutanol was added, and was upregulated in the mutant strain Sc12. *DIP5* and *GNP1* were up-regulated when isobutanol was added. *Avt7* was up-regulated when WT and Sc12 were under isobutanol stress. **Figure S9.** Expression level of the *ACC1* gene. *ACC1* is the rate-limiting step for de novo fatty acid biosynthesis. It was up-regulated in WT under isobutanol stress. When the tryptophan pathway was defective, the expression of *ACC1* was down-regulated under isobutanol stress but could be recovered by adding external tryptophan. **Figure S10.** Expression level of the *TAT2* gene. *TAT2* is a tryptophan amino acid transporter and was up-regulated when WT and Sc12 were under isobutanol stress. **Table S1.** Genes related to isobutanol tolerance. **Table S2.** Tophat mapping rate of NGS reads. **Table S3.** Gene names and numbers used in clustering analysis.

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## Authors' contributions
H-LL and CH-TW designed the study, performed the experiments and analyzed the data. H-LL drafted the manuscript. E-PIC contributed to data interpretation and manuscript revision. W-HL and C-CH coordinated and advised the study and revised the manuscript. All authors read and approved the final manuscript.

## Availability of data and materials
The datasets supporting the conclusions of this article are included in the article and its additional files. The NGS data generated in the present study are available in the NCBI repository (GSE175794).

## Declarations

### Ethics approval and consent to participate
Not applicable.

### Consent for publication
Not applicable.

### Competing interests
The authors declare no competing interests.

### Author details
[1]Program in Microbial Genomics, National Chung Hsing University and Academia Sinica, Taipei, Taiwan. [2]Biodiversity Research Center, Academia Sinica, 128 Academia Road, Sec. 2, Taipei 115, Taiwan. [3]Department of Life Sciences, National Chung Hsing University, No. 145, Xingda Rd., Taichung 402, Taiwan. [4]Department of Food Science and Biotechnology, National Chung Hsing University, No. 145, Xingda Rd., Taichung 402, Taiwan. [5]Department of Ecology and Evolution, University of Chicago, Chicago, IL 60637, USA. [6]Innovation and Development Center of Sustainable Agriculture, National Chung Hsing University, No. 145, Xingda Rd., Taichung 402, Taiwan.

## References
1. Promdonkoy P, Siripong W, Downes JJ, Tanapongpipat S, Runguphan W. Systematic improvement of isobutanol production from d-xylose in engineered Saccharomyces cerevisiae. AMB Express. 2019;9:1–14.
2. Brownstein AM. Renewable motor fuels: the past, the present and the uncertain future. Oxford: Butterworth-Heinemann; 2014.
3. Avalos JL, Fink GR, Stephanopoulos G. Compartmentalization of metabolic pathways in yeast mitochondria improves the production of branched-chain alcohols. Nat Biotechnol. 2013;31:335–41.
4. Matsuda F, Ishii J, Kondo T, Ida K, Tezuka H, Kondo A. Increased isobutanol production in *Saccharomyces cerevisiae* by eliminating competing pathways and resolving cofactor imbalance. Microb Cell Fact. 2013;12:1–11.
5. Choi YJ, Lee J, Jang YS, Lee SY. Metabolic engineering of microorganisms for the production of higher alcohols. MBio. 2014. https://doi.org/10.1128/mBio.01524-14.
6. Deparis Q, Claes A, Foulquié-Moreno MR, Thevelein JM. Engineering tolerance to industrially relevant stress factors in yeast cell factories. FEMS Yeast Res. 2017. https://doi.org/10.1093/femsyr/fox036.
7. Liu HL, Chang JJ, Thia C, Lin YJ, Lo SC, Huang CC, Li WH. Characterizing an engineered carotenoid-producing yeast as an anti-stress chassis for building cell factories. Microb Cell Factories. 2019;18:1–9.
8. Chin WC, Lin KH, Chang JJ, Huang CC. Improvement of n-butanol tolerance in Escherichia coli by membrane-targeted tilapia metallothionein. Biotechnol Biofuels. 2013;6:1–9.
9. Chin WC, Lin KH, Liu CC, Tsuge K, Huang CC. Improved n-butanol production via co-expression of membrane-targeted tilapia metallothionein and the clostridial metabolic pathway in *Escherichia coli*. BMC Biotechnol. 2017;17:1–14.
10. Kuroda K, Hammer SK, Watanabe Y, López JM, Fink GR, Stephanopoulos G, Ueda M, Avalos JL. Critical roles of the pentose phosphate pathway and GLN3 in isobutanol-specific tolerance in yeast. Cell Syst. 2019;9:534–47.
11. Dittmar JC, Reid RJ, Rothstein R. ScreenMill: a freely available software suite for growth measurement, analysis and visualization of high-throughput screen data. BMC Bioinform. 2010;11:1–11.
12. González-Ramos D, van den Broek M, van Maris AJ, Pronk JT, Daran JMG. Genome-scale analyses of butanol tolerance in Saccharomyces cerevisiae reveal an essential role of protein degradation. Biotechnol Biofuels. 2013;6:1–18.

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13. Stanley D, Bandara A, Fraser S, Chambers PJ, Stanley GA. The ethanol stress response and ethanol tolerance of *Saccharomyces cerevisiae.* J Appl Microbiol. 2010;109:13–24.

14. Cheng Y, Du Z, Zhu H, Guo X, He X. Protective effects of arginine on *Saccharomyces cerevisiae* against ethanol stress. Sci Rep. 2016;6:1–12.

15. Creutz CE. Expression of metazoan annexins in yeast provides protection against deleterious effects of the biofuel isobutanol. Sci Rep. 2019;9:1–6.

16. Schroeder L, Ikui AE. Tryptophan confers resistance to SDS-associated cell membrane stress in *Saccharomyces cerevisiae.* PloS ONE. 2019;14:e0199484.

17. Xu K, Liu G, Fu C. The tryptophan pathway targeting antioxidant capacity in the placenta. Oxid Med Cell Longev. 2018. https://doi.org/10.1155/2018/1054797.

18. Thomas SR, Stocker R. Redox reactions related to indoleamine 2,3-dioxygenase and tryptophan metabolism along the kynurenine pathway. Redox Rep. 1999;4:199–220.

19. Xiao W, Wang RS, Handy DE, Loscalzo J. NAD (H) and NADP (H) redox couples and cellular energy metabolism. Antioxid Redox Signal. 2018;28:251–72.

20. Rao RP, Hunter A, Kashpur O, Normanly J. Aberrant synthesis of indole-3-acetic acid in Saccharomyces cerevisiae triggers morphogenic transition, a virulence trait of pathogenic fungi. Genetics. 2010;185:211–20.

21. Prusty R, Grisafi P, Fink GR. The plant hormone indoleacetic acid induces invasive growth in *Saccharomyces cerevisiae.* PNAS. 2004;101:4153–7.

22. Bramucci MG, Larossa RA, Singh M. Yeast with increased butanol tolerance involving filamentous growth response. US Patent No. 8,557,562. 15 Oct 2013.

23. Liu YY, Chen HW, Chou JY. Variation in indole-3-acetic acid production by wild *Saccharomyces cerevisiae* and *S. paradoxus* strains from diverse ecological sources and its effect on growth. PLoS ONE. 2016;11:e0160524.

24. Perli T, Wronska AK, Ortiz-Merino RA, Pronk JT, Daran JM. Vitamin requirements and biosynthesis in *Saccharomyces cerevisiae.* Yeast. 2020;37:283–304.

25. Crespo JL, Powers T, Fowler B, Hall MN. The TOR-controlled transcription activators GLN3, RTG1, and RTG3 are regulated in response to intracellular levels of glutamine. PNAS. 2002;99:6784–9.

26. Brauer MJ, Yuan J, Bennett BD, Lu W, Kimball E, Botstein D, Rabinowitz JD. Conservation of the metabolomic response to starvation across two divergent microbes. PNAS. 2006;103:19302–7.

27. Kowalska E, Kujda M, Wolak N, Kozik A. Altered expression and activities of enzymes involved in thiamine diphosphate biosynthesis in *Saccharomyces cerevisiae* under oxidative and osmotic stress. FEMS Yeast Res. 2012;12:534–46.

28. Beas AO, Gordon PB, Prentiss CL, Olsen CP, Kukurugya MA, Bennett BD, Parkhurst SM, Gottschling DE. Independent regulation of age associated fat accumulation and longevity. Nat Commun. 2020;11:1–10.

29. Koo YS, Kim H, Park JH, Kim MJ, Shin YI, Choi BT, Lee SY, Shin HK. Indoleamine 2,3-dioxygenase-dependent neurotoxic kynurenine metabolism contributes to poststroke depression induced in mice by ischemic stroke along with spatial restraint stress. Oxid Med Cell Longev. 2018. https://doi.org/10.1155/2018/2413841.

30. Sun Y. Indoleamine 2,3-dioxygenase–a new antioxidant enzyme. Mater Med Pol. 1989;21:244–50.

31. Patel MS, Mireille J-B, Ballard FJ, Hanson RW. Pathway of carbon flow during fatty acid synthesis from lactate and pyruvate in rat adipose tissue. J Lipid Res. 1971;12:179–91.

32. Hirasawa T, Yoshikawa K, Nakakura Y, Nagahisa K, Furusawa C, Katakura Y, Shimizu H, Shioya S. Identification of target genes conferring ethanol stress tolerance to Saccharomyces cerevisiae based on DNA microarray data analysis. J Biotechnol. 2007;131:34–44.

33. Abe F, Horikoshi K. Tryptophan permease gene TAT2 confers high-pressure growth in *Saccharomyces cerevisiae.* Mol Cell Biol. 2000;20:8093–102.

34. Sherman BT, Tan Q, Collins JR, Alvord WG, Roayaei J, Stephens R, Baseler MW, Lane HC, Lempicki RA. The DAVID gene functional classification tool: a novel biological module-centric algorithm to functionally analyze large gene lists. Genome Biol. 2007;8:1–16.

35. Metsalu T, Vilo J. ClustVis: a web tool for visualizing clustering of multivariate data using principal component analysis and heatmap. Nucleic Acids Res. 2015;43:W566–70.

36. Kolde R, Kolde MR. Package 'pheatmap'. R package. 2015;1:790.

37. Babicki S, Arndt D, Marcu A, Liang Y, Grant JR, Maciejewski A, Wishart DS. Heatmapper: web-enabled heat mapping for all. Nucleic Acids Res. 2016;44:W147–53.

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